

Developmental Regulation and Activity-Dependent Maintenance of GABAergic Presynaptic Inhibition onto Rod Bipolar Cell Axonal Terminals

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SUMMARY

Presynaptic inhibition onto axons regulates neuronal output, but how such inhibitory synapses develop and are maintained in vivo remains unclear. Axon terminals of glutamatergic retinal rod bipolar cells (RBCs) receive GABA_A and GABA_C receptor-mediated synaptic inhibition. We found that perturbing GABAergic or glutamatergic neurotransmission does not prevent GABAergic synaptogenesis onto RBC axons. But, GABA release is necessary for maintaining axonal GABA receptors. This activity-dependent process is receptor subtype specific: GABA_C receptors are maintained, whereas GABA_A receptors containing $\alpha 1$, but not $\alpha 3$, subunits decrease over time in mice with deficient GABA synthesis. GABA_A receptor distribution on RBC axons is unaffected in GABA_C receptor knockout mice. Thus, GABA_A and GABA_C receptor maintenance are regulated separately. Although immature RBCs elevate their glutamate release when GABA synthesis is impaired, homeostatic mechanisms ensure that the RBC output operates within its normal range after eye opening, perhaps to regain proper visual processing within the scotopic pathway.

INTRODUCTION

Presynaptic inhibition onto axonal terminals, commonly provided by GABAergic transmission, regulates neurotransmitter release. GABA receptors on the axon hillock of pyramidal cells (Nusser et al., 1996; Szabadics et al., 2006), on mossy fiber terminals of hippocampal granule cells (Ruiz et al., 2003), on cerebellar parallel fibers (Stell et al., 2007), and on retinal bipolar cell axon terminals (Shields et al., 2000; Vardi and Sterling, 1994) serve to modulate action potential firing and neurotransmitter release (Kullmann et al., 2005; Luscher et al., 2011). Although much is known about how presynaptic GABAergic inhibition

shapes neuronal output, mechanisms that regulate the development and maintenance of such inhibition onto axon terminals are not yet well understood. Here, we addressed the role of neurotransmission in the development, maturation, and maintenance of GABAergic synapses onto axonal terminals involved in modulation of neurotransmitter release. To do so, we took advantage of a well-characterized circuit in the mammalian retina, where glutamate release from axons is regulated by robust presynaptic GABAergic inhibition.

At dim light levels, visual information from rod photoreceptors is conveyed to rod bipolar cells (RBCs), which relay the signal to amacrine cells in the inner retina (reviewed by Wässle, 2004). Specifically, RBCs contact GABAergic A17 amacrine cells (A17s) (Nelson and Kolb, 1985), which provide local feedback inhibition onto the rod bipolar cell axon terminals to modulate their release of glutamate (Chávez et al., 2006; Chun et al., 1993; Hartveit, 1999). In addition, RBC axon terminals also receive GABAergic drive from other widefield amacrine cells (Chávez et al., 2010; McGuire et al., 1984). Unlike other parts of the brain in which GABAergic inhibition is mediated mainly by GABA_A receptors, GABAergic input onto RBC axon terminals involves both GABA_A and GABA_C receptor types (Enz et al., 1996; Koulen et al., 1998). GABA_A and GABA_C receptors do not cocluster at the same synaptic site but are expressed at different postsynaptic sites on RBC axon terminals (Fletcher et al., 1998; Koulen et al., 1998). There are also functional differences between the two receptor types: GABA_A receptors are less sensitive to GABA but have faster kinetics compared to GABA_C receptors, and thus the two receptor types regulate different aspects of glutamate release from RBCs (Eggers and Lukasiewicz, 2006b; Sagdullaev et al., 2006). The diversity of GABA receptors on RBCs presents an ideal opportunity to investigate how neurotransmission regulates the development and maintenance of not only GABA receptors but also different GABA receptor types on the same axon terminal.

To examine the role of neurotransmission on the formation and maintenance of axonal GABA receptors, we utilized transgenic mice in which GABAergic or glutamatergic transmission is perturbed throughout the period of circuit assembly. Using electron microscopy, immunohistochemistry, and electrophysiology, we assessed whether perturbed neurotransmission affects the

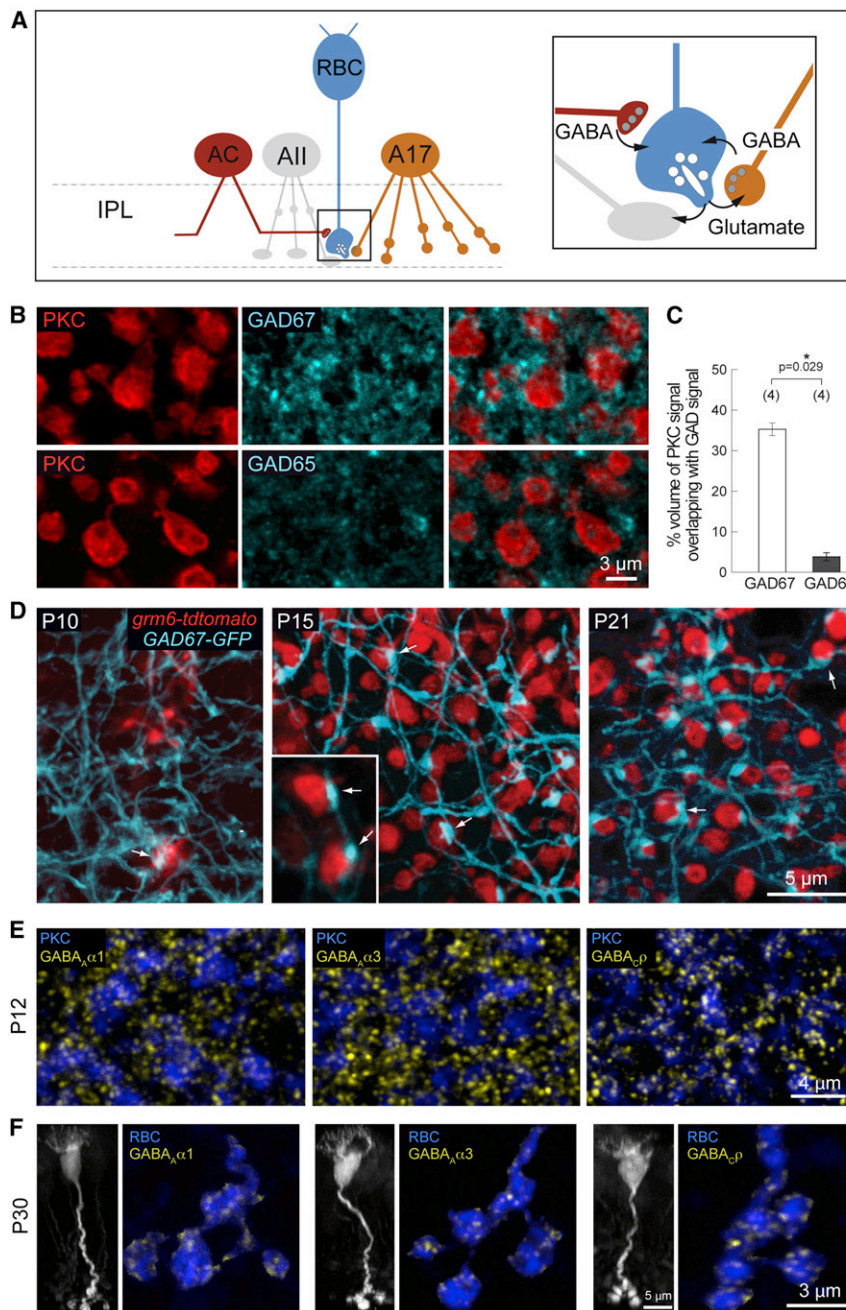


Figure 1. Development of GABAergic Synapses onto RBC Axon Terminals

(A) Schematic showing RBC connections with amacrine cells in retina IPL. RBCs (blue) provide excitatory glutamatergic drive onto glycinergic AII (gray) and GABAergic A17 (orange) amacrine cells. In turn, they receive feedback inhibition from A17 cells and additional GABAergic input from other as-yet-unidentified amacrine cells (AC; red).

(B) PKC-labeled RBC terminals (red) colabeled with GAD67 or GAD65 (cyan) reveal more abundant immunoreactivity of GAD67 as compared to GAD65 in the IPL lamina, where RBC axons stratify.

(C) Quantification of percent volume of GAD signal overlapping with PKC immunoreactivity revealed a significantly higher percent overlap with GAD67-positive processes as compared to GAD65. Asterisk marks significant difference.

(D) Development of contacts (arrows) between RBCs (red) and GAD67-GFP-positive amacrine processes (cyan) in the *grm6-tdtomato* \times *GAD67-GFP* double transgenic line. By P15, large varicosities can be seen at sites of contact between RBC axon terminals and GAD67-GFP amacrine processes.

(E) Axonal terminals of P12 PKC-labeled RBCs (blue) express GABA α 1, GABA α 3, and GABA ρ receptor clusters (yellow), indicating the presence of these three GABAergic postsynapses on RBC terminals before eye opening.

(F) P30 RBCs in the *grm6-tdtomato* transgenic line (left, vertical view, grayscale). Axonal terminals of individual P30 RBCs (horizontal view, blue) express all three GABA receptor cluster types: GABA α 1, GABA α 3, and GABA ρ (yellow). See also Figures S1 and S2.

whether lack of GABAergic transmission also caused developmental changes in the glutamatergic synapses of A17 amacrine cells.

RESULTS

Development of GABAergic Synapses on RBC Axon Terminals

GABAergic inhibition onto axon terminals of RBCs is mediated by a variety of amacrine cells, including A17 cells that provide reciprocal feedback inhibition (Figure 1A). GABA is synthesized in amacrine cells by two isoforms of glutamate decarboxylase, GAD67 and GAD65 (Haverkamp and Wässle, 2000; Vardi and Auerbach, 1995). We found that in the sublamina of the inner plexiform layer (IPL) where RBC axonal terminals stratify (sublamina 5, ON-layer), GAD67 immunoreactivity was more abundant compared to GAD65 (Figure 1B). Quantification of the immunolabeling (see Experimental Procedures) showed that dendritic processes positive for GAD67 exhibited greater volume overlap with protein kinase C (PKC) immunoreactive RBC axonal boutons, compared

structural formation and maintenance of GABA receptor subtypes on RBC axon terminals. Because RBC terminals also receive some glycinergic inhibition (Eggers et al., 2007; Wässle et al., 2009), we further analyzed whether an upregulation of glycine receptors occurred to compensate for the reduction in GABAergic transmission. Furthermore, we asked whether the loss of GABAergic inhibition causes alterations to RBC output beyond that expected solely from disinhibition in the GABA-deficient circuit. Because GABA release from A17 amacrine cells normally requires RBC drive due to the reciprocal synaptic arrangement between these two cell types, we determined

whether lack of GABAergic transmission also caused developmental changes in the glutamatergic synapses of A17 amacrine cells. GABA is synthesized in amacrine cells by two isoforms of glutamate decarboxylase, GAD67 and GAD65 (Haverkamp and Wässle, 2000; Vardi and Auerbach, 1995). We found that in the sublamina of the inner plexiform layer (IPL) where RBC axonal terminals stratify (sublamina 5, ON-layer), GAD67 immunoreactivity was more abundant compared to GAD65 (Figure 1B). Quantification of the immunolabeling (see Experimental Procedures) showed that dendritic processes positive for GAD67 exhibited greater volume overlap with protein kinase C (PKC) immunoreactive RBC axonal boutons, compared

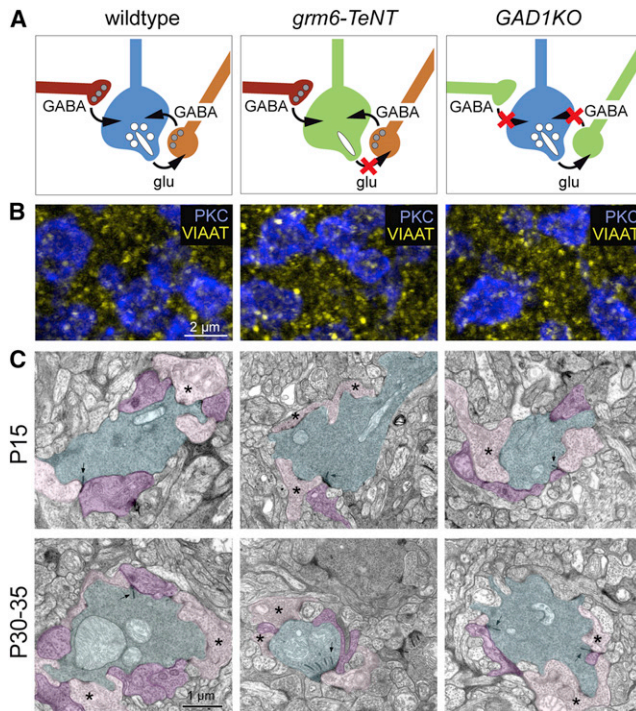


Figure 2. Inhibitory Synapses onto RBC Axons in *grm6-TeNT* and *GAD1KO* Retina

(A) Schematic showing RBC axon terminal circuitry in wild-type and transgenic lines with suppressed glutamatergic (glu) transmission from ON-bipolar cells (*grm6-TeNT*) and reduced GABAergic transmission from amacrine cells (*GAD1KO*).

(B) Immunolabeling for VIAAT (yellow) at the level of PKC-positive RBC boutons (blue) in P30 wild-type, *grm6-TeNT*, and *GAD1KO* retina.

(C) Electron micrographs showing dyad synapses between RBC boutons (cyan) and All (purple) and A17 (pink) amacrine cell processes in the wild-type, *grm6-TeNT*, and *GAD1KO* retinae at P15 and P30–P35. Presynaptic ribbons are indicated by arrows. Asterisks mark examples of A17 contact. Note that RBCs in the P30 *grm6-TeNT* retina have multiple ribbons at a single dyad synapse.

See also Figure S3.

to GAD65 containing processes (Figure 1C), suggesting that GAD67-positive amacrine cell processes provide the majority of GABAergic inhibition onto RBC axon terminals.

To visualize amacrine cell contacts onto RBC terminals during development, we utilized the *GAD67-GFP* transgenic line (Chattopadhyaya et al., 2004), where a fraction of GAD67-positive amacrine cells (Figure S1A available online) express GFP. Amacrine cells typically synapse onto bipolar cell axons at enlarged varicosities (Dowling and Boycott, 1966). Using the *GAD67-GFP* transgenic line, we visualized large varicosities of GFP-labeled amacrine processes contacting RBC boutons at sites immunopositive for GAD67, but not GAD65 (Figure S1B). We further determined when GABAergic contacts develop on RBCs, labeled by expression of the fluorescent protein tdTomato under the *grm6* promoter (Figure 1D). Appositions between GAD67-GFP positive processes and RBC axonal boutons were already apparent at postnatal day (P) 10 (Figure 1D), several days after axonal differentiation in the bipolar cells (Morgan

et al., 2006). At P15, large varicosities were clearly present at sites of contact between amacrine cell dendrites and RBC boutons, which remained evident at P21. We previously found that spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) can be recorded from RBCs from P10 (Schubert et al., 2008). Thus, the appearance of structural contacts between GABAergic amacrine cells and RBC axon terminals coincides with the emergence of sIPSCs in RBCs. This suggests that functional postsynaptic GABA receptor clusters are already present on RBC terminals before eye opening (P15).

Previous studies demonstrated that GABA_A and GABA_C receptors are present on axon terminals of RBCs across species (Fletcher et al., 1998; Koulen et al., 1997, 1998; Lukasiewicz, 1996, 2004; Enz et al., 1996; McCall et al., 2002). To monitor the appearance of GABA receptor clusters on RBC axonal boutons during circuit assembly, we labeled for GABA_A and GABA_C receptors at two developmental time points, before eye opening (P12) and at maturity (P30). We found abundant GABA_C receptor clusters on PKC-labeled axon terminals of RBCs as early as P12 and at P30 (Figures 1E and 1F). To determine the subunit composition of the GABA_A receptor clusters located at these terminals, we performed immunostaining for GABA_A α 1– α 3 subunits together with PKC. α 2-containing GABA_A receptors were not present on RBC axon terminals (Figure S2A), supporting past findings (Fletcher et al., 1998). In contrast, both α 1- and α 3-containing GABA_A receptor clusters were localized on RBC axonal boutons at both ages examined (Figures 1E and 1F). GABA_A synapses containing α 1 or α 3 subunits did not colocalize with GABA_C receptors (Figure S2B) as found previously in adult retina (Koulen et al., 1996; Wässle et al., 1998). Furthermore, these three GABA receptor cluster types (α 1-GABA_A, α 3-GABA_A, and GABA_C) on RBC boutons were each apposed to large GAD67-GFP amacrine cell varicosities (Figure S1C). Coimmunolabeling for GAD67 and GABA receptors revealed that 93.27% \pm 0.48% of GABA_A α 1 (n = 2 animals), 93.89% \pm 0.66% of GABA_C (n = 2), and 79.30% \pm 2.04% of GABA_A α 3 (n = 3) receptor clusters on RBC terminals were apposed to GAD67-positive terminals. Thus, all three GABA receptor types are present on RBC terminals before eye opening, suggesting that synapses comprising these receptor types develop concurrently.

Synaptic Contacts between RBCs and Amacrine Cells Form Despite Reduction of GABAergic or Glutamatergic Neurotransmission

To determine the role of neurotransmission in the structural development of inhibitory synapses onto RBC axon terminals, we used two transgenic mouse lines (Figure 2A). First, we asked whether suppressing glutamatergic transmission onto amacrine cells that likely contact RBCs affects the development of amacrine cell synapses onto RBC axons. This was achieved using the *grm6-TeNT* line in which neurotransmission from all ON-bipolar cells, including RBCs, is suppressed by expression of the light chain of tetanus toxin (Kerschensteiner et al., 2009). Next, we assessed the outcome when GABA release from amacrine cells is diminished from development onward. To do so, we crossed *GAD1* conditional knockout (KO) mice (Chattopadhyaya et al., 2004) with α Pax6-Cre mice in which cre-recombinase expression is limited to the retina, to generate *GAD1KO* mice

(Schubert et al., 2010) (Figure S3). In *GAD1KO* retina, the level of GAD67 expression is reduced (Figure S3A), and immunolabeling for GAD67 reveals that loss of GAD67 (in the knockout region) occurs outside a dorsal-ventral strip (the WT region) in which cre-recombinase is largely absent (Figures S3B and S3C; see Marquardt et al., 2001). We use “*GAD1KO*” from here on to refer to the knockout regions.

We compared vesicular inhibitory amino acid transporter (VIAAT) immunolabeling across wild-type, *grm6-TeNT*, and *GAD1KO* retinas to assess whether amacrine cell terminals surrounding RBC axons failed to differentiate when neurotransmission is perturbed. We found no gross changes in the density of VIAAT labeling surrounding P30 RBC axonal terminals in either transgenic line, suggesting that amacrine cell-RBC synapses still formed (Figure 2B). We confirmed that amacrine cell-RBC synapses still formed in the mutant mice, by examining the ultrastructural arrangement of RBC axonal boutons in both *grm6-TeNT* and *GAD1KO* retina. In both lines, amacrine cells still synapsed onto RBC boutons, and these synapses were apparent at eye opening (P15) ($n = 14$ *GAD1KO*; $n = 26$ *TeNT* RBC boutons examined) as in wild-type animals (Figure 2C). However, some aspects of the synaptic arrangements differed between *grm6-TeNT* and *GAD1KO* retinas. P30 RBC boutons formed dyad synapses at sites containing a single ribbon in the *GAD1KO* ($n = 20$ RBC synapses per genotype), but such arrangements were disrupted in *grm6-TeNT* retinas, where sometimes multiple ribbons were apposed to a single postsynaptic density ($n = 16$ of 26 at P15, and 13 of 23 synaptic sites had >1 ribbon at P30; e.g., Figure 2C), as shown previously (Kerschensteiner et al., 2009). Thus, although the ribbon arrangement of the RBC is differentially affected in *grm6-TeNT* and *GAD1KO* retinas, the amacrine cell-RBC synapse forms and is maintained regardless of whether bipolar or amacrine cell transmission is perturbed.

Development and Maintenance of GABAergic Synapses on RBC Axon Terminals Do Not Rely on Glutamatergic Transmission

Although amacrine cell synapses are present structurally on RBC terminals in *grm6-TeNT* retinas, it is possible that suppression of glutamatergic drive from bipolar cells leads to alterations in GABA-mediated transmission onto RBC axon terminals. We thus performed whole-cell recordings on wild-type and *grm6-TeNT* RBCs and analyzed spontaneous GABAergic inhibitory postsynaptic currents (sIPSCs) (Figure 3A). Spontaneous IPSCs in both genotypes were comparable in mean frequency and amplitude at both P11–P13 and P30, indicating that the number of GABAergic synaptic contacts and the size of postsynaptic GABA receptor clusters were not significantly affected by a reduction of glutamatergic input to amacrine cells (Figure 3B). To evoke release of GABA from presynaptic amacrine cells, we puffed α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) at the axon terminal of RBCs (Chávez et al., 2006) and recorded the evoked PSCs at 0 mV holding potential (Figure 3C). The mean charge of AMPA-evoked currents was not different in wild-type and *grm6-TeNT* RBCs at both P11–P13 and P30 (Figure S4). To distinguish GABA_A and GABA_C components of the evoked response, AMPA puffs were repeated in the presence of (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid (TPMPA)

(GABA_C receptor antagonist) or SR95531 (GABA_A receptor antagonist) (Figure 3C). Quantification of mean charge and amplitude (data not shown) of the evoked response (total, GABA_A, or GABA_C) showed no significant differences between RBCs in wild-type and *grm6-TeNT* retinas at P11–P13 (Figure 3D) and at P30 (Figure 3E). Together, these results suggest that functional GABAergic synapses are formed normally and maintained even when the bipolar cells fail to transmit effectively to amacrine cells.

RBC Responses to GABA Application Decline with Maturation in the *GAD1* Mutant

To assess the functional consequences of reduced GABA synthesis on the formation and maintenance of inhibitory synapses onto RBC axon terminals, we performed whole-cell recordings of these neurons in the *GAD1KO* retina. As expected, spontaneous inhibitory postsynaptic currents (sIPSCs) were rare in *GAD1KO* at both ages examined (Figures 4A and 4B). This indicates that deletion of GAD67 was not accompanied by a compensatory upregulation of the other GABA synthesizing enzyme, GAD65. Because synaptic release of GABA in these mutants is greatly impaired, we puffed GABA onto *GAD1KO* RBC axon terminals and recorded the evoked chloride currents in order to assess whether any postsynaptic changes occurred in *GAD1KO* RBCs. Interestingly, RBC responses to GABA application in *GAD1KO* were unchanged at P11–P13 but were dramatically reduced at P30 (example recordings in Figure 4C). Both mean amplitude and charge of the evoked responses decreased by P30 (Figure 4D). Moreover, the rise time was slower for evoked responses in P30 *GAD1KO* RBCs, whereas their decay time was faster, as compared to control (Figure 4E). Thus, GABAergic transmission plays an important role in the maintenance, although not in the initial formation, of functional GABAergic synapses on RBC axon terminals.

In wild-type animals, RBCs receive little glycinergic input (Eggers et al., 2007). Indeed, immunolabeling for the $\alpha 1$ or $\alpha 2$ subunit of the glycine receptor (Ivanova and Müller, 2006; Wässle et al., 2009) showed little glycine receptor expression on RBC axon terminals (Figures S5A and S5B). However, a severe reduction of GABAergic transmission in *GAD1KO* may be compensated for by an upregulation of glycine receptor-driven input onto RBC axon terminals. We investigated this possibility by quantifying the expression of glycine receptors containing $\alpha 1$ or $\alpha 2$ subunits on RBC axon terminals in *GAD1KO* retina. But, we did not find any upregulation of these glycine receptor subtypes in the RBC terminals of *GAD1KO* (Figure S6).

GABA_A and GABA_C Receptors on RBC Axon Terminals Are Differentially Altered in *GAD1KO*

Because GABA_A and GABA_C receptor clusters are found at distinct postsynaptic sites on RBC axon terminals, we next asked whether loss of GABAergic transmission affected both receptor types. Figure 5A shows examples of the GABA-evoked responses of P30 RBCs from a littermate control and a *GAD1KO* animal in which the GABA_A component is revealed upon blocking the GABA_C receptor-mediated current. Quantification of the mean amplitude and charge of the evoked GABA_A responses in RBCs revealed significant reduction in the knockout animal (Figure 5B). Similarly, the evoked GABA_C responses were

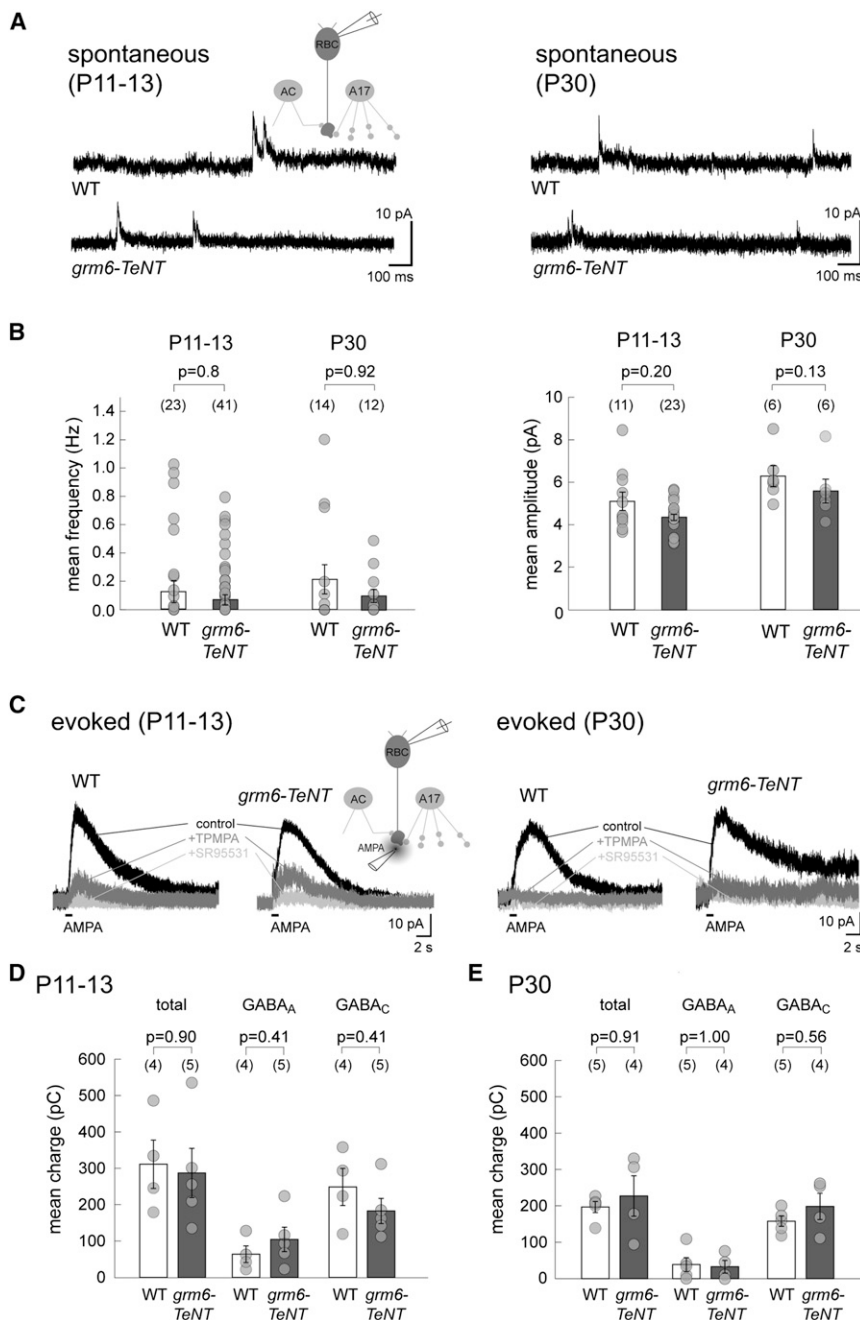


Figure 3. Spontaneous and GABA-Evoked Currents of RBCs in *grm6-TeNT* Retina

(A) Spontaneous GABAergic currents recorded in *grm6-TeNT* and wild-type (WT) RBCs at P11–P13 and P30.

(B) Quantification revealed no significant differences in either the mean frequency or mean amplitude of GABAergic sIPSCs from *grm6-TeNT* RBCs as compared to WT.

(C) Example traces showing chloride-mediated outward currents in RBCs evoked by AMPA puffs in *grm6-TeNT* and WT RBCs at both ages. The GABA_C component was isolated by application of TPMPA and residual GABA_A-mediated current blocked by SR95531.

(D and E) Scatter plots show that the mean charge transfers of the total, GABA_A-mediated, and GABA_C-mediated currents are comparable between *grm6-TeNT* and WT RBCs at P11–P13 (D) and P30 (E). Bracketed numbers in (B), (D), and (E) represent the number of cells. Error bars represent SEM.

See also Figure S4.

units of the GABA_C receptors. We compared the immunolabeling of P30 knockout regions in the *GAD1* mutant with corresponding wild-type regions (which provides an ideal control because these regions are within the same retina) as well as with littermate control retinas. For GABA_A receptors, GAD67 immunostaining was used to distinguish knockout regions from wild-type regions in *GAD1KO* (Figures 6A and 6B). However, we could not colabel GAD67 and GABA_C receptors due to species specificity of the antibodies. Instead, we used the GFP signal to identify the knockout regions because GFP is expressed specifically in cells in which the *GAD1* exon is excised (Marquardt et al., 2001). Overall, immunoreactivity for $\alpha 1$ -containing GABA_A receptors was significantly reduced in the knockout region compared to the wild-type region and littermate control (Figure 6A). In contrast, $\alpha 3$ -containing GABA_A receptor labeling

isolated for RBCs in *GAD1KO* and control upon blocking GABA_A currents (Figure 5C). In contrast to GABA_A-mediated responses, the mean amplitude of the GABA_C-mediated response was unchanged (Figure 5D). However, the net charge carried by the GABA_C currents was significantly reduced in *GAD1* (Figure 5D). This may reflect faster GABA_C-mediated response kinetics in RBCs from *GAD1KO* compared to littermate control (see Figure 4E).

To correlate these functional changes at P30 with the expression of GABA_A and GABA_C receptor types, we immunostained for the $\alpha 1$ and $\alpha 3$ subunits of the GABA_A receptors and the ρ sub-

did not appear to have changed in the knockout regions (Figure 6B). Similarly, GABA_C receptor staining was comparable across regions and genotypes (Figure 6C). Because of the high density of GABA receptor clusters on RBC boutons, it was not always possible to separate individual clusters. Thus, instead of determining the number of receptor puncta, we quantified the percent volume occupied by each receptor subtype on PKC-positive RBC boutons (see Experimental Procedures). We found that the percent volume occupied by $\alpha 1$ -containing GABA_A receptors, but not $\alpha 3$ -containing GABA_A receptors or GABA_C receptors, was significantly reduced in the knockout

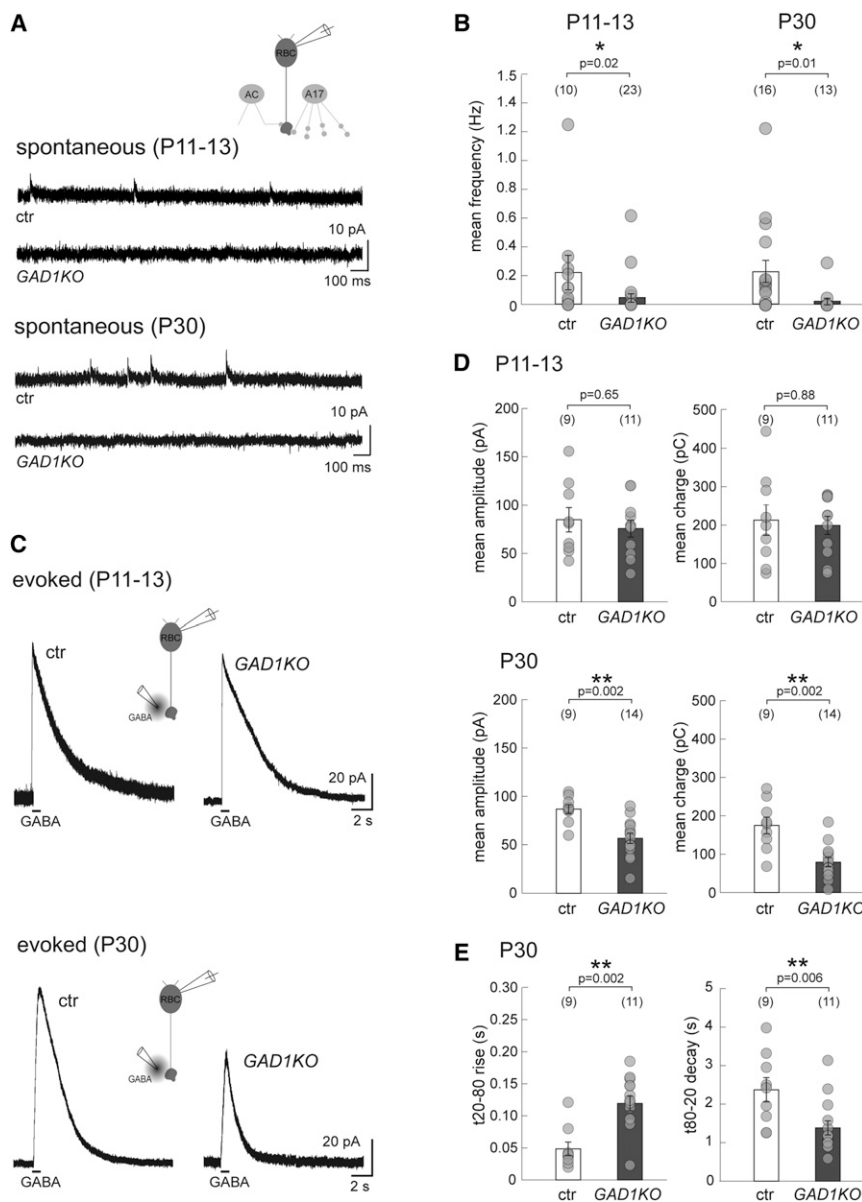


Figure 4. Spontaneous and GABA-Evoked Currents of RBCs in *GAD1KO* Retina

(A) Spontaneous GABAergic IPSCs at RBC axon terminals recorded from *GAD1KO* and littermate control (ctr) at P11–P13 and P30.

(B) Scatter plots show that the mean frequency of these sIPSCs is reduced in *GAD1KO* as compared to ctr.

(C) Puffs of GABA evoke similar chloride-mediated outward currents in RBCs from ctr and *GAD1KO* at P11–P13, which are markedly reduced for *GAD1KO* RBCs at P30.

(D) Quantification reveals a significant reduction for both the mean peak amplitude and charge of GABA-evoked currents from *GAD1KO* RBCs at P30.

(E) In addition, the rise time for the P30-evoked response from *GAD1KO* RBCs is longer, whereas the decay is faster as compared to control. Bracketed numbers in (B), (D), and (E) represent the number of cells. Asterisks mark significant difference. Error bars represent SEM.

See also Figure S3.

regions (Figure 6). This reduction of GABA α 1 clusters in GAD67-deficient regions was corroborated by using another GABA α 1 antibody raised in a different species (Figure S6A). To assess whether GABA α 1 synthesis levels in *GAD1KO* retina was diminished overall, we performed western blot analysis using P30 retina homogenates from which the dorsal-ventral wedge was removed. The total GABA α 1 synthesis levels in the *GAD1KO* mutant was comparable to littermate control samples (Figure S6B; $n = 2$ *GAD1KO* and 2 littermate controls), suggesting that overall synthesis of GABA α 1 receptors in GAD67-deficient retina was unchanged. The reduction in immunostaining for GABA α 1 in the *GAD1KO* is thus unlikely to be caused by an alteration in receptor synthesis and is more likely the result of a deficit in the maintenance of GABA α 1 clusters on RBC terminals. Because we found little expression of the known inhibitory

postsynaptic scaffolding proteins, Neuro-ligin2 and Gephyrin, at GABA α receptors in RBC axon terminals (Figures S5B and S5C), we decided against analyzing the expression levels of these proteins in *GAD1KO* RBCs. Taken together, our findings demonstrate that GABA α and GABA γ receptors on RBC axon terminals are differentially maintained by GABAergic transmission.

The specific reduction of GABA α and not GABA γ receptor clusters in *GAD1KO* RBCs suggests an independent regulation of the maintenance of these two ionotropic GABA receptor types that coexist on the same axonal terminal. To further test this hypothesis, we immunolabeled α 1-containing GABA α receptor clusters in *GABA γ KO* mice (McCall et al., 2002) (Figure S7). We found no

alteration in the percent volume occupied by α 1-containing GABA α receptors in P30 *GABA γ KO* RBC terminals (Figure S7). Thus, we conclude that there exists independent regulatory mechanisms for maintaining GABA α and GABA γ receptor clusters on RBC axon terminals.

Reduced GABAergic Drive Transiently Increases Glutamate Release from Developing RBCs

GABAergic A17 amacrine cells form reciprocal synapses with glutamatergic RBCs. We thus wondered whether there were corresponding changes in the RBC input onto the A17 cells in *GAD1KO*. We compared RBC input onto A17 cells in *GAD1KO* with wild-type littermate controls at two developmental time points, P11–P13 and P30, by recording spontaneous excitatory postsynaptic currents (sEPSCs) from A17 amacrine cells. In

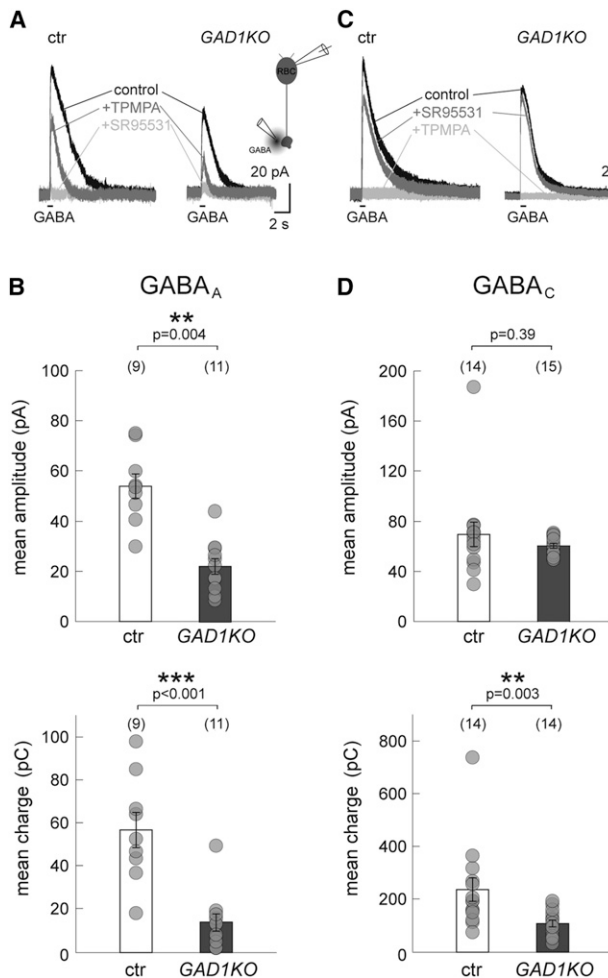


Figure 5. GABA_A and GABA_C Receptor-Mediated Currents from P30 RBCs in *GAD1KO* Retina

(A) Example traces of P30 *GAD1KO* and littermate control (ctr) RBC responses to GABA puffs. TPMPA was applied to isolate the GABA_A-mediated component. Further addition of SR95531 blocked all evoked response.

(B) Scatter plots show that mean peak amplitude and charge of GABA_A-mediated currents in *GAD1KO* RBCs are significantly reduced as compared to ctr. (C) Application of SR95531 alone isolates the GABA_C-mediated component of evoked responses in RBCs.

(D) Quantification of GABA_C-mediated currents indicates a significant reduction of the mean amplitude and charge of *GAD1KO* RBCs as compared to ctr. However, the mean amplitude remained unchanged. Bracketed numbers in (B) and (D) represent the number of cells. Asterisks mark significant difference. Error bars represent SEM.

See also Figure S3.

littermate controls, the mean frequency of sEPSCs in these amacrine cells normally increased with age (Figures 7A and 7B). In *GAD1KO*, the mean frequency of sEPSCs from A17 amacrine cells at P11–P13 was significantly higher compared to their control littermates (Figures 7A and 7B). In the KO, there was no further increase in sEPSCs frequency in the A17 cells with maturation (Figures 7A and 7B), and in fact, the P11–P13 sEPSCs were already comparable to that of P30 controls. These observations together suggest that although the RBC output is initially

elevated during development when inhibition is reduced, homeostatic mechanisms ensure that the bipolar cell output operates within its normal range at maturity (Figure 7B).

Is the transient increase in frequency of the sEPSCs from developing A17 cells in the KO simply a result of reduced inhibition onto the RBC axonal terminals? To answer this, we recorded sEPSCs from A17s in P11–P13 *GAD1KO* and littermate control animals in the presence of GABA_A and GABA_C receptor blockers, SR95531 and TPMPA, respectively (Figure 7C). Even in the presence of GABA receptor blockers, P11–P13 A17 cells showed an increased frequency of sEPSCs in *GAD1KO* as compared to control (Figure 7C), suggesting that there is an upregulation of glutamatergic drive onto A17 cells in *GAD1KO*. This upregulation, however, is not the consequence of perturbed GABA_C-receptor mediated transmission, despite GABA_C receptors carrying the majority of the total charge transfer (Figures 5B and 5D; Eggers and Lukasiewicz, 2006a; McCall et al., 2002). In the presence of a GABA_A receptor antagonist, the mean sEPSC frequency of P11–P13 A17 cells in the GABA_C receptor KO is not significantly different from that of littermate controls (Figure S8).

Could the upregulation of glutamatergic drive onto developing A17 cells be due to changes in receptor density on A17 cells? We found that at P11–P13, the mean amplitude of the sEPSCs was unchanged for A17s in *GAD1KO* (Figures 7D and 7E), indicating that the glutamate receptor density at individual postsynaptic sites is not altered. Also, A17 cell responses to AMPA puffs revealed no differences between *GAD1KO* and control (Figures 7F and 7G). Thus, the total density (or number) of glutamatergic synapses on A17s is unperturbed in *GAD1KO* animals (Figures 7D–7G). This suggests that the increase in A17 sEPSC mean frequency in *GAD1KO* is not the result of changes in glutamate receptor density on the A17 cell but is more likely due to presynaptic changes, such as the probability of release, in the RBC terminal.

DISCUSSION

We found that RBC axon terminals receive GABAergic inhibition from GAD67-positive amacrine cells via three distinct GABA receptor subtypes (GABA_Aα1, GABA_Aα3, and GABA_C). Using mutant mice, we found that GABAergic synapses are still established on RBC axonal terminals when either glutamatergic or GABAergic transmission is perturbed. However, the maintenance of GABA_Aα1 receptor clusters on RBC axonal terminals is selectively disturbed when GABA synthesis is much reduced in the presynaptic amacrine cells (Figure 8). Further, the maintenance of GABA_Aα1 receptor clusters is not dependent on the presence or synaptic drive via GABA_C receptors. We also discovered that glutamate release from developing RBCs increased in the *GAD1KO*, but not in the GABA_CKO retinas (Figure 8).

GABAergic Synapse Maintenance, but Not Formation, on Axon Terminals Is Influenced by GABAergic Transmission

How neurotransmission modulates the formation of inhibitory synapses has primarily been addressed for synapses onto

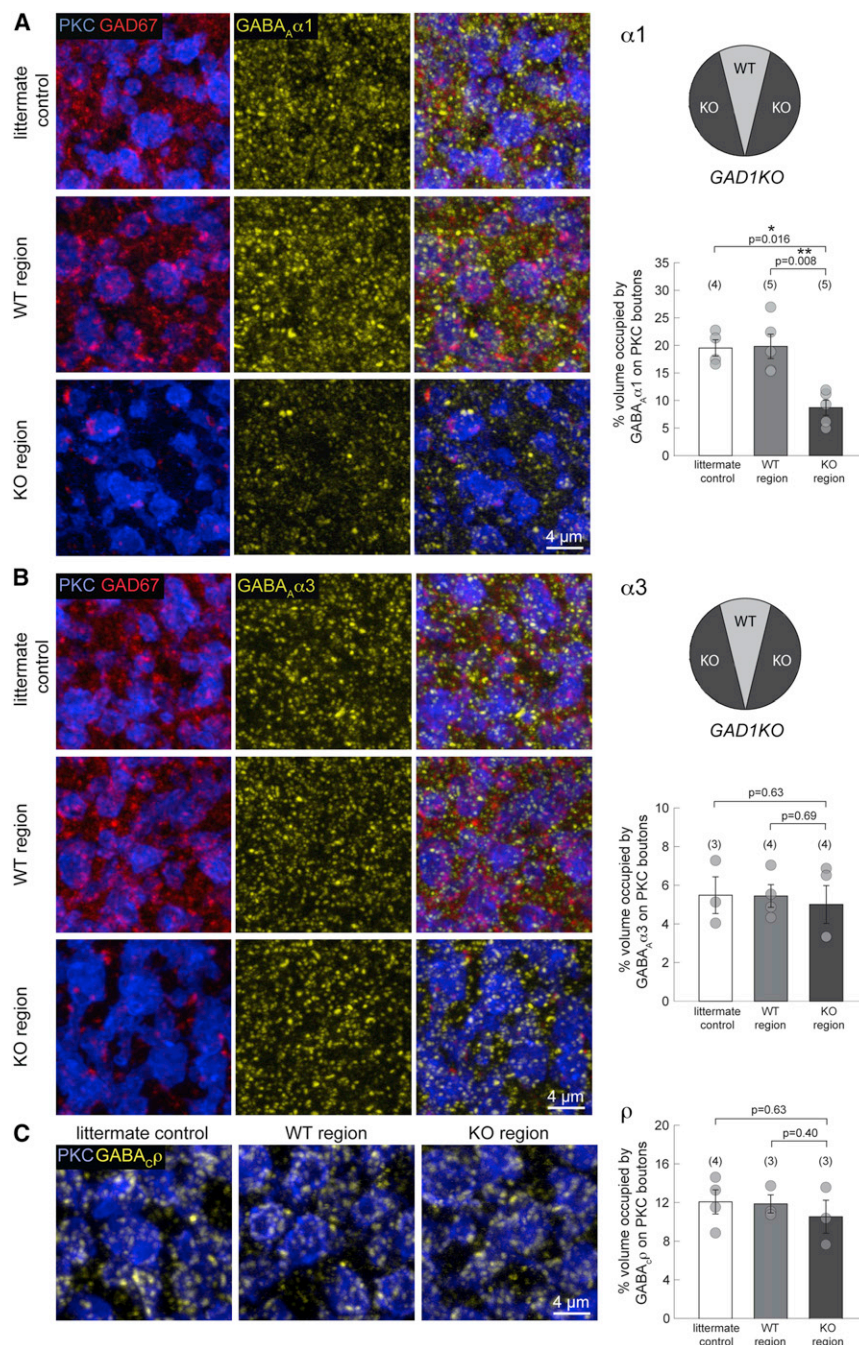


Figure 6. GABA_A Receptor Subsets on Mature RBC Boutons in *GAD1KO* Retina

(A) Triple immunolabeling of PKC expressing RBCs (blue), GAD67 (red), and GABA_A $\alpha 1$ subunits (yellow) revealed a visible reduction of $\alpha 1$ -containing GABA_A receptor clusters in the KO region of *GAD1KO* retina, compared to the WT region or littermate control. Quantification of the percent volume occupied by GABA_A $\alpha 1$ clusters on PKC-positive boutons confirmed a significant reduction in the KO region.

(B) Immunostaining for the GABA_A $\alpha 3$ receptor subunit revealed no significant differences in the percent volume occupied by these receptor clusters on KO region RBC boutons.

(C) Immunolabeling and percent volume occupancy of GABA_A ρ receptor subunits were comparable across littermate control, WT regions, and KO regions. Bracketed numbers represent the number of animals. Asterisks mark significant difference. Error bars represent SEM. See also Figures S3, S5, S6, and S7.

GABA release onto RBCs as well as GABA receptor density of RBCs are unchanged in the *grm6-TenT* retinas. This is in contrast to a reduced number of inhibitory synapses and decrease in synaptic vesicle density of terminals contacting the dendrites of spinal cord neurons cultured in the presence of glutamate (non-NMDA) receptor antagonists (Rosato-Siri et al., 2002).

Prior work has demonstrated that GABAergic transmission is not essential for inhibitory synapse formation on dendrites and somas per se (Chattopadhyaya et al., 2007; Wojcik et al., 2006; Wu et al., 2012). We found this to also be true for inhibitory synapse formation onto axon terminals as amacrine cell-RBC synapses are evident in the retinal-specific *GAD1KO*. Reduction of GAD67 in basket cells of the visual cortex, however, results in fewer perisomatic inhibitory synapses on pyramidal neurons (Chattopadhyaya et al., 2007). This reduction in inhibitory synapse number onto the cell bodies appears to

somata and dendrites of neurons (Chattopadhyaya et al., 2007; Hartman et al., 2006; Harms and Craig, 2005; Kilman et al., 2002). Recently, GABAergic transmission was found to regulate the maturation of basket interneuron axonal terminals (Fu et al., 2012). Here, we assessed the importance of neurotransmission in the development of GABAergic synapses on glutamatergic axon terminals, focusing on amacrine cell-RBC connectivity. Our results demonstrate that presynaptic GABAergic amacrine cells do not require glutamatergic drive from bipolar cells to establish their inhibitory synapses onto bipolar cell axons.

be due to the lack of GABAergic transmission during synaptogenesis, rather than a failure to maintain established synapses. In the retina, we found that reducing GABAergic transmission during development affects the maintenance of GABA receptors on the RBC axons, but not the initial formation of these synapses. From previous work (Burrone and Murthy, 2003; Pozo and Goda, 2010; Turrigiano, 2007), we had expected that RBCs in *GAD1KO* might undergo homeostatic adjustment and recruit more GABA receptors to their axons to compensate for reduced GABAergic transmission.

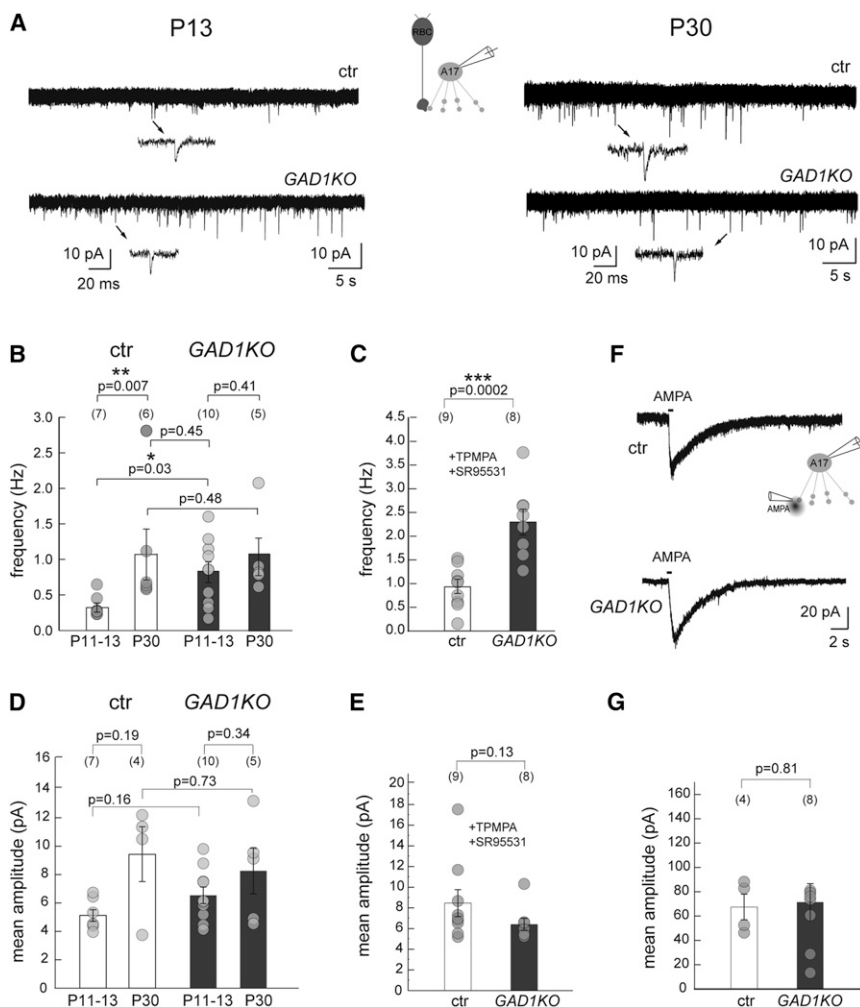


Figure 7. RBC Output Is Transiently Increased in *GAD1KO* Retina during Circuit Development

(A) sEPSCs recorded from A17 amacrine cells at P13 and P30 in *GAD1KO* and littermate control (ctr) retina show an increase during normal development.

(B) The sEPSC frequency was increased for A17 amacrine cells in *GAD1KO* as compared to ctr early in development at P13 but was comparable to control at P30.

(C) Mean frequency of sEPSCs from P11–P13 A17 amacrine cells in *GAD1KO* and ctr in the presence of GABA receptor antagonists TPMPA and SR95531 also show increased frequency in the *GAD1KO*.

(D) Mean sEPSC amplitudes recorded in A17s from littermate control and *GAD1KO* at P11–P13 and P30 reveal no differences.

(E) Mean amplitude of sEPSCs recorded from P11–P13 A17 amacrine cells in *GAD1KO* and ctr in the presence of GABA receptor antagonists TPMPA and SR95531 show no difference.

(F) Example traces showing AMPA-evoked cation-mediated inward currents from A17 cells clamped at -60 mV in ctr and *GAD1KO* at P11–P13.

(G) Scatter plots show that the mean amplitude is comparable between genotypes. Bracketed numbers represent the number of cells. Asterisk marks significant difference. Error bars represent SEM.

See also Figures S3 and S8.

Instead, we observed that RBC axon terminals lose GABA receptors at maturity when presynaptic GABA release is reduced chronically.

Activity-Dependent Maintenance of Axonal GABA Receptors Is Receptor Subtype Specific

To date, most studies focusing on the activity-dependent maintenance of GABA_A receptors in neurons have assessed the distribution of the entire GABA_A receptor population, irrespective of their subunit composition. This is because in most parts of the nervous system, GABA_A receptors can comprise mixed α subunits together with β and γ subunits (Fritschy and Mohler, 1995; Kasugai et al., 2010). However, in the mammalian retina, three distinct subtypes of GABA_A receptors can be distinguished by the presence of specific α subunits ($\alpha 1$ – $\alpha 3$) localized at nonoverlapping synapses (Koulen et al., 1996; Wässle et al., 1998). On mouse RBC axon terminals, we identified two types of GABA_A receptor synapses, containing either the $\alpha 1$ or $\alpha 3$ subunit. Both these GABA_A receptor types were apposed to GAD67-positive processes but, functionally, they could provide GABA_A receptor-mediated inhibition with different time courses, because $\alpha 1$ -containing GABA_A receptors exhibit faster response

kinetics compared to $\alpha 3$ -containing receptors (Gingrich et al., 1995; Ortinski et al., 2004; Vicini et al., 2001). Surprisingly, we found that reduced GABAergic neurotransmission selectively regulated the maintenance of GABA_A $\alpha 1$, but not GABA_A $\alpha 3$, receptor clusters.

Reducing GABAergic transmission also differentially affected the maintenance of the two ionotropic GABA receptor types (GABA_A and GABA_C) on RBC axons. Whereas the amplitude of the GABA_A receptor component of the GABA-evoked response was decreased at P30 in *GAD1KO*, there was no change in amplitude of the GABA_C component. Our immunostaining for GABA_A and GABA_C receptors supported this differential reduction in GABA_A versus GABA_C receptors on RBC axons. What might be the differences in GABA_A and GABA_C receptors that could account for this differential outcome in the *GAD1KO*? GABA_C receptors have a higher affinity for GABA compared to GABA_A receptors (Feigenspan and Bormann, 1994). Perhaps these receptors require only low levels of GABA for their maintenance. GABA_A and GABA_C receptors do not colocalize at the same postsynaptic sites (Fletcher et al., 1998; Koulen et al., 1998). Thus, it could also be that these two GABA receptor types are differentially positioned on the RBC terminal relative to the GABA release site, such that GABA_A receptors normally “sense” higher GABA levels compared to GABA_C receptors and thus need a substantial amount of GABA in the synaptic cleft for their

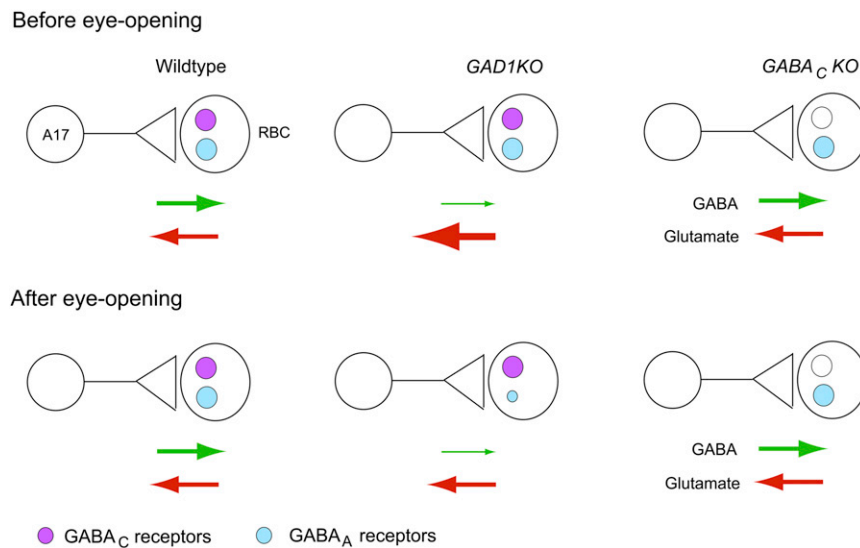


Figure 8. Summary of the Alterations in RBC-A17 Synapses in GABAergic Transmission-Defective Mutants before and after Eye Opening

Schematic illustrating the A17-RBC synapse before and after eye opening in wild-type, $GAD1KO$, and $GABA_C KO$ retina. GABA and glutamate release are similar between wild-type and $GABA_C KO$ (see also Eggers and Lukasiewicz, 2006a) but is altered in $GAD1KO$. There is a transient upregulation of glutamate release from developing RBCs in $GAD1KO$ before eye opening. However, after eye opening, glutamate release from RBCs in $GAD1KO$ becomes comparable to wild-type. $GABA_A$, and not $GABA_C$, receptors are selectively reduced at maturity in $GAD1KO$. This reduction of $GABA_A$ receptors on RBCs does not occur in $GABA_C$ receptor mutants.

maintenance. We further showed that loss of $GABA_C$ receptors per se does not affect the maintenance of $GABA_A\alpha1$ receptors in RBC terminals. We found no downregulation of $GABA_A$ receptors in $GABA_C KO$ retina. Furthermore, in $GABA_C KO$ retina, the function of glycine receptors (Eggers and Lukasiewicz, 2006a) on RBCs axon terminals is not affected. Accordingly, in $GAD1KO$ we found no upregulation of glycine receptor clusters on RBC axon terminals. Taken together, our observations highlight independent mechanisms for regulating the distribution and density of distinct inhibitory receptor types (GABA receptors versus glycine receptors, $GABA_A$ versus $GABA_C$ receptors, and $GABA_A\alpha1$ versus $GABA_A\alpha3$ receptor types) on the same RBC axon terminal.

What underlies the eventual reduction of $\alpha1$ -containing $GABA_A$ receptors in $GAD1KO$? The role of activity in inhibitory receptor accumulation has been addressed in spinal cord cultures, where blocking neuronal activity by tetrodotoxin (TTX) application prevented glycine receptor accumulation (Kirsch and Betz, 1998). However, our physiological recordings suggest that GABA receptors accrue normally on RBC axons initially (P11–P13), and total $GABA_A\alpha1$ synthesis in adult (P30) $GAD1KO$ retina also appeared unimpaired. Another possibility is a failure to stabilize $GABA_A\alpha1$ receptor clusters after they have formed at synapses (Saliba et al., 2007). Tracking movements of the inhibitory postsynaptic scaffold protein gephyrin in dissociated spinal cord neurons previously demonstrated activity-dependent stabilizations of the gephyrin-fluorescent protein conjugates at synaptic versus extrasynaptic locations (Hanus et al., 2006). The phenotypic alterations we observed in density of $\alpha1$ -containing $GABA_A$ receptors on RBC axons in $GAD1KO$ could be a result of failed stabilizations of $GABA_A$ receptors at this synapse. This could lead to greater receptor internalization and subsequent degradation detectable as reduced receptor volume occupancy. Indeed, blocking neuronal activity has been shown previously to induce ubiquitination and degradation of $GABA_A$ receptors (Luscher et al., 2011). However, because Neuroligin2 and Gephyrin do not appear to be at all

$GABA_A\alpha1$ receptor clusters on the RBCs, future work is necessary to identify the postsynaptic scaffold proteins at the RBC axonal GABA receptors before we can systematically investigate the molecular mechanisms underlying the loss of $GABA_A\alpha1$ receptor clusters on RBC axon terminals in the $GAD1$ mutant.

RBCs Adjust Their Output Homeostatically in Response to Reduced GABAergic Drive

We found that RBCs increase their output onto A17 amacrine cells with maturation. Our study shows that this output is structurally and functionally modified by alterations in neurotransmission during development and circuit maturation. When glutamate release from RBCs is suppressed by TeNT expression in these cells, more ribbons are often recruited to RBC output sites. When GABAergic transmission is impaired, glutamate release from developing RBC terminals is increased beyond that expected solely from disinhibition in the GABA-deficient circuit, perhaps as an attempt to facilitate GABA release from the “silenced” A17 amacrine cells with which it shares a reciprocal synapse. Interestingly, this increase in glutamate release in developing RBCs in $GAD1KO$ retinas does not appear to evoke a change in glutamate receptor density on the A17 amacrine cell. The lack of a complementary change in the A17 cell is unexpected for two reasons: (1) the RBC-A17 synapse is bidirectional, and (2) in other systems, downregulation of postsynaptic glutamate receptors occurs when there is enhanced presynaptic glutamate release due to homeostatic mechanisms coming into play (Burrone and Murthy, 2003; Pozo and Goda, 2010; Turrigiano, 2007). In $GAD1KO$, RBC output further undergoes homeostatic regulation with maturation to “cap” or limit its output with circuit maturation, perhaps to maintain normal processing in the scotopic pathway involving the other postsynaptic partner, the AII amacrine cell (Bloomfield and Dacheux, 2001). This later homeostatic regulation of RBC output parallels the loss of $GABA_A$ receptors on RBCs, but these events are unlikely to be related. This is because the progressive loss of

GABA receptors does not affect inhibition on RBCs as there is little GABA release in *GAD1KO*. Our results highlight divergent mechanisms behind the regulation of output from RBC axon terminals and GABA receptor maintenance on these terminals. Our current findings also underscore the importance of assessing changes in GABA receptors on axons in addition to the somata and dendrites of neurons for understanding neurodevelopmental disorders, such as schizophrenia, where GABAergic transmission is perturbed (Lewis et al., 2005; Wassef et al., 2003).

EXPERIMENTAL PROCEDURES

Transgenic Mouse Lines

Several transgenic mouse lines were used in this study. In the *GAD67-GFP* (G42) line, the *GAD67* promoter drives GFP expression in a fraction of GABAergic amacrine cells (Chattopadhyaya et al., 2004). In the *grm6-tdtomato* mouse line, the red fluorescent protein *tdtomato* is expressed specifically by ON-bipolar cells under the mGluR6 promoter (Kerschensteiner et al., 2009). In *GAD1/lox/lox* mice (Chattopadhyaya et al., 2007), exon 2 of *GAD1*, the gene encoding GAD67 (Bu et al., 1992), is flanked by loxP sites. To obtain retina-specific excision of exon 2, this line was bred to the α -Cre line, in which Cre-recombinase expression is regulated by the alpha enhancer of the *Pax6* promoter (Marquardt et al., 2001). The excision of exon 2 in cells expressing Cre-recombinase results in a frameshift mutation of *GAD1* (Chattopadhyaya et al., 2007). We refer to this double transgenic line as *GAD1KO*. To abolish glutamatergic transmission in RBCs, we used *grm6-TeNT* transgenic in which the light chain of tetanus toxin is expressed specifically in ON-bipolar cells (Kerschensteiner et al., 2009). To eliminate GABA_C receptors from the retina, the gene encoding GABA_C1 subunit was inactivated (McCall et al., 2002); we refer to these mice as *GABA_CKO*.

Immunohistochemistry

Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Washington. All procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice deeply anesthetized with Isoflurane were decapitated and enucleated. The cornea was punctured with a 30 gauge needle, and the retinas were removed in cold oxygenated mouse artificial cerebrospinal fluid (mACSF [pH 7.4]) containing (in mM) 119 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1 NaH₂PO₄, 11 glucose, and 20 HEPES. For vibratome sectioning, the retina was fixed for 20 min in 4% paraformaldehyde in mACSF (pH 7.4). For fixed flat-mount preparations, retinas were isolated and mounted retinal ganglion cell side up on black membrane filters (HABP013, Millipore, Billerica, MA, USA). The retina and filter paper were then immersed in 4% paraformaldehyde in mACSF (pH 7.4) for either 15 min (for GABA_Aα1 and GABA_Aα3 immunolabeling) or 30 min (for GABA_C labeling). After fixation, the tissue was washed in 0.1 M PBS (pH 7.4), preincubated in PBS containing 5% normal donkey serum (NDS) and 0.5% Triton X-100, and incubated with primary antibodies in the same solution. For retinal whole-mounts, primary antibody incubation was performed over three nights. Secondary antibody incubation was carried out in PBS, and retinas were subsequently mounted in Vectashield (Vector Labs, Burlingame, CA, USA). Immunolabeling was performed using antibodies against PKC (rabbit polyclonal, 1:1,000, Chemicon, Temecula, CA, USA, or mouse monoclonal, 1:1,000, Sigma-Aldrich, St. Louis), GAD67 (mouse monoclonal, 1:1,000, Chemicon), GAD65 (rabbit polyclonal, 1:1,000, Chemicon), VIAAT (guinea pig polyclonal, 1:1,000, Synaptic Systems, Goettingen, Germany), GFP (chicken polyclonal, 1:1,000, Abcam, Cambridge, UK), Glycine receptor α1 subunit (mouse monoclonal mAb2b, 1:500, Synaptic Systems), Glycine receptor α2 subunit (goat polyclonal, 1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Gephyrin (mouse monoclonal 3B11, 1:1,000, Synaptic Systems), Neuroligin2 (rabbit polyclonal, 1:4,000, kindly provided by F. Varoqueaux and N. Brose), GABA_Cρ receptor subunit (rabbit polyclonal, 1:500, kindly provided by R. Enz), GABA_Aα1 receptor subunit (polyclonal guinea-pig, 1:5,000, kindly provided by J.M. Fritschy), GABA_Aα1 receptor

subunit (polyclonal rabbit, 1:2,000, kindly provided by J.M. Fritschy), GABA_Aα2 receptor subunit (polyclonal guinea-pig, 1:2,000, kindly provided by J.M. Fritschy), and GABA_Aα3 receptor subunit (polyclonal guinea-pig, 1:3,000, kindly provided by J.M. Fritschy). Secondary antibodies utilized were either anti-isotypic Alexa Fluor conjugates (1:1,000, Invitrogen, Carlsbad, CA, USA) or DyLight conjugates (1:1,000, Jackson ImmunoResearch, West Grove, PA, USA).

Imaging and Analysis

Image stacks were acquired on an Olympus FV1000 laser scanning confocal microscope. Fixed tissue was imaged using a 1.35 NA 60× oil immersion objective at a voxel size of 0.069, 0.069, 0.3 μm or 0.051, 0.051, 0.3 μm (x, y, z) for images used in quantification. To visualize RBC-amacrine cell contacts at different developmental time points, we crossed the *GAD67-GFP* and *grm6-tdtomato* mouse lines and imaged the retinas using a custom-built two-photon microscope and an Olympus 60× water objective. Each optical plane was averaged three to four times. Raw image stacks were processed using MetaMorph (Molecular Devices, Sunnyvale, CA, USA) and Amira (Visualization Sciences Group, Burlington, MA, USA). For volume estimation, the PKC signal of RBC boutons was masked in three dimensions using the “label field” function of Amira. Subsequently, this PKC mask was multiplied with the GAD or GABA receptor channel to isolate signal specifically within the RBC boutons. Next, a constant threshold for image stacks from the same retina (including WT-KO region comparison across the retina), was applied to detect the volume occupied by the signal using the “label voxel” function of Amira. The percent of signal volume as compared to PKC mask volume was estimated using the “tissue statistics” function of Amira.

The specificity of GABA receptor antibodies utilized in this study was tested by performing colocalization analysis and assessing the extent of random associations (Soto et al., 2011). In vertical retinal sections, we found 87% of GABA_Aα3 receptor clusters apposed to the presynaptic marker, VIAAT.

Electron Microscopy

Retinas were fixed by eyecup immersion in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 3 hr. The tissue was then washed in buffer, fixed further in 1% osmium tetroxide in cacodylate buffer for 1 hour prior to en bloc staining with 1% uranyl acetate. The tissue was then dehydrated in graded ethanol series, embedded in araldite resin, sectioned, and stained with 5% lead citrate.

Western Blot

Retinas from *GAD1KO* and littermate control mice (two retinas pooled per animal) were prepared on ice in homogenizing buffer (50 mM TRIS, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail. Samples (30 μg/ml per lane) were run by SDS-PAGE (10% resolving gel), blotted onto nitrocellulose membranes, incubated with antibodies, and visualized with enhanced chemiluminescence. Primary antibodies utilized were directed against GAD67 (mouse monoclonal, 1:2,000, Chemicon), GABA_Aα1 receptor subunit (polyclonal rabbit, 1:2,000, kindly provided by J.M. Fritschy), or actin (mouse monoclonal, 1:3,000, Chemicon). For preparation of samples to compare GABA_Aα1 levels from retinal homogenates, the dorsal-ventral wedge of the retina was not included for both *GAD1KO* and littermate control samples.

Preparation of Mouse Retinal Slices for Whole-Cell Recording

The preparation of retinal slices has been described in detail by Eggers and Lukasiewicz (2006a). In brief, the eyecup was incubated for 20 min in mACSF containing 0.5 mg/ml hyaluronidase (Sigma-Aldrich) to remove any remaining vitreous. The retinas were then put onto filter paper (HABP013, Millipore) with the photoreceptor layer facing up. Vertical retinal slices of 200 μm thickness were cut using a standard technique (Werblin, 1978) and subsequently stored in carbogenated mACSF at room temperature.

Whole-Cell Recordings and Data Analysis

Whole-cell recordings were made from RBCs (in *GAD1KO* and *grm6-TeNT*) and A17s (in *GAD1KO* and *GABA_CKO*) in retinal slices as previously described (Eggers and Lukasiewicz, 2006a; Schubert et al., 2008). The resistance of the

electrodes with standard solutions usually ranged between 6 and 8 M Ω . Liquid junction potentials of 15 mV were corrected before measurements with the pipette offset function. Series resistance (mean: 67.6 ± 3.7 M Ω , $n = 18$) and capacitance of pipettes as well as cell capacitance were not compensated. Seal resistances >1 G Ω were routinely obtained. To record GABA-evoked currents and spontaneous inhibitory postsynaptic currents (sIPSCs) in RBCs, we voltage-clamped these cells at 0 mV, the reversal potential for cation-mediated currents through ionotropic glutamate receptors. For all RBC recordings, glycinergic input and network activity were blocked by strychnine (0.5 μ M) and TTX (1 μ M), respectively. For A17 amacrine cells, excitatory postsynaptic currents mediated by ionotropic glutamate receptors (sEPSCs) were recorded at a holding potential of -60 mV, the reversal potential for chloride-mediated currents set by the internal solution (Schubert et al., 2008). Action potentials in A17s were blocked by including QX-314 (2 mM) in the intracellular solution. For recording sEPSCs from P11–P13 A17s in the presence of GABA receptor blockers, TPMPA (50 μ M) and SR95531 (5 μ M) were included in the extracellular mACSF solution. The holding current was monitored during the experiment, and recordings with shifting holding currents were aborted. All experiments were carried out at room temperature (20°C–22°C) and under room light conditions.

Data acquisition was performed using an Axopatch 200B amplifier with an acquisition frequency of 10 kHz using the Clampex software (Axon Instruments, Foster City, CA, USA). Signals were 2 kHz Bessel filtered. Data were further processed using a routine written in Igor Pro 6 (Wavemetrics, Portland, OR, USA) and visualized with Origin software (Microcal, Northampton, MA, USA). To quantify parameters of spontaneous synaptic events, the mean frequency \pm SEM and the mean peak amplitude \pm SEM of the events were determined. To quantify evoked currents, peak amplitude and total charge transfer (as complete area under the curve), both relative to the preapplication baseline current, were determined and averaged across cells. In addition, we characterized the kinetics of the evoked current by determining the time points from the exponential fits at which the traces reached 20% and 80% of the peak amplitude and calculated the differences, t_{20-80} rise and t_{80-20} decay.

Solution and Drugs

The extracellular solution used for recording (mACSF) contained (in mM) 122.5 NaCl, 5 KCl, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 2 CaCl₂, and 20 glucose, adjusted to pH 7.4 with 95% O₂ and 5% CO₂. The intracellular solution contained (in mM) 120 cesium gluconate, 1 CaCl₂, 1 MgCl₂, 10 Na-HEPES, 11 EGTA, and 10 TEA-Cl and was adjusted to pH 7.2 with CsOH. Under our experimental conditions, the calculated chloride equilibrium potential was -59.3 mV. To block GABA_C receptors and GABA_A receptors, (1,2,5,6-tetrahydropyridine-4-yl) methyphosphinic acid (TPMPA, 50 μ M) and SR95531 (5 μ M) were used, respectively. All drugs were purchased from Sigma-Aldrich. Solutions in the recording chamber were exchanged using a gravity-driven superfusion system previously described (Lukasiewicz and Roeder, 1995). GABA receptor and glutamate receptor agonists, GABA (200 μ M) and AMPA (100 μ M), were dissolved in mACSF with 0.005% sulforhodamine B and applied with a puff pipette (5–7 M Ω) using a Picospritzer system. Puffing directions, as well as the duration of the 300 ms puff, were chosen such that the axonal terminal of the patched RBC was completely covered by the puff, visualized by including sulforhodamine B in the puffer pipette.

Statistics

For comparisons across data sets, the Wilcoxon rank-sum test was used in all cases. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2013.01.037>.

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